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(54) **Streptococcus pneumoniae polypeptide greA**

(57) greA polypeptides and DNA (RNA) encoding such greA and a procedure for producing such polypeptides by recombinant techniques is disclosed. Also disclosed are methods for utilizing such greA for the treatment of infection, particularly bacterial infections. Antagonists against such greA and their use as a therapeutic

to treat infections, particularly bacterial infections are also disclosed. Also disclosed are diagnostic assays for detecting diseases related to the presence of greA nucleic acid sequences and the polypeptides in a host. Also disclosed are diagnostic assays for detecting polynucleotides encoding greA/greB gene family and for detecting the polypeptide in a host.

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Description

This invention relates, in part, to newly identified polynucleotides and polypeptides; variants and derivatives of these polynucleotides and polypeptides; processes for making these polynucleotides and these polypeptides, and their variants and derivatives; agonists and antagonists of the polypeptides; and uses of these polynucleotides; polypeptides, variants, derivatives, agonists and antagonists. In particular, in these and in other regards, the invention relates to polynucleotides and polypeptides of greA/greB gene family, hereinafter referred to as "greA"

BACKGROUND OF THE INVENTION

The Streptococci make up a medically important genera of microbes known to cause several types of disease in humans, including, for example otitis media, conjunctivitis, pneumonia, bacteremia, meningitis, sinusitis, pleural empyema and endocarditis, and most particularly meningitis, such as for example infection of cerebrospinal fluid. Since its isolation more than 100 years ago, *Streptococcus pneumoniae* has been one of the more intensively studied microbes. For example, much of our early understanding that DNA is, in fact, the genetic material was predicated on the work of Griffith and of Avery, MacLeod and McCarty using this microbe. Despite the vast amount of research with *S. pneumoniae*, many questions concerning the virulence of this microbe remain. It is particularly preferred to employ Streptococcal genes and gene products as targets for the development of antibiotics.

Transcription of DNA is often arrested at sites in DNA that trap a fraction of elongating RNA polymerase molecules that pass through, resulting in locked ternary complexes that cannot propagate or dissociate their RNA product. Transcript cleavage factors cleave the RNA in such complexes at the 3'-end, allowing RNA polymerase to back up and re-attempt to read through the potential trap (Borukhov et al. 1993, Cell 72:459-466). In addition to assuring efficient transcript elongation, transcript cleavage factors increase the fidelity of transcription since misincorporated bases at the 3'-end of the nascent RNA also lead to arrested complexes (Erie et al. Science 262:867-8730). Two transcript cleavage factors, greA and greB, have been identified in *E. coli* (Borukhov et al. 1993, Cell 72:459-466). greA-dependent transcript cleavage usually results in the removal of di- and trinucleotides from the 3'-end of the stalled RNA. greB-dependent cleavage yields larger oligonucleotides, up to a length of nine nucleotides. Both proteins bind RNA polymerase. Neither the greA or greB proteins possess intrinsic nuclease activity rather they stimulate a nuclease activity inherent in RNA polymerase (Orlova et al. 1995, Proc. Natl. Acad. Sci., USA 92:4596-4600). The greA and greB proteins are homologous, sharing 38% sequence identity and 59% sequence similarity. The eukaryotic transcript elongation protein SII is similar to the greA and greB proteins in that it stimulates RNA cleavage from the 3'-end of RNA in a stalled complex but does not share significantly sequence homology with the greA and greB proteins (Borukhov et al. 1993, Cell 72:459-466).

greA and greB proteins and their homologues, such as the novel polypeptides and polynucleotides of the invention, are important for screening for and discovery of antimicrobial compounds, particularly to fill the unmet medical need of developing new classes of such antimicrobial compounds.

Clearly, there is a need for factors, such as greA/greB homologue of the invention, that may be used to screen compounds for antibiotic and antimicrobial activity and which may also be used to determine their roles in pathogenesis of infection, dysfunction and disease. There is a need, therefore, for identification and characterization of such factors which can play a role in preventing, ameliorating or correcting infections, dysfunctions or diseases.

The polypeptide of the present invention has sequence homology to a known prokaryotic transcript cleavage factor protein.

SUMMARY OF THE INVENTION

Toward these ends, and others, it is an object of the present invention to provide polypeptides, *inter alia*, that have been identified as novel greA peptides by homology between the amino acid sequence set out in Figure 2 [SEQ ID NO:2] and known amino acid sequences of other proteins such as *E. coli* greA protein.

It is a further object of the invention, moreover, to provide polynucleotides that encode novel greA polypeptides, particularly polynucleotides that encode the polypeptide herein designated greA.

In a particularly preferred embodiment of this aspect of the invention the polynucleotide comprises the region encoding novel greA polypeptides in the sequence set out in Figure 1 [SEQ ID NO:1] or a fragment, analogue or derivative thereof.

In another particularly preferred embodiment of the present invention there is a novel prokaryotic transcript cleavage factor protein from *Streptococcus pneumoniae* comprising the novel amino acid sequence of Figure 2 [SEQ ID NO:2], or a fragment, analogue or derivative thereof.

In accordance with this aspect of the present invention there is provided an isolated nucleic acid molecule encoding a mature polypeptide expressible by the bacterial strain *Streptococcus pneumoniae* 0100993 contained in NCIMB

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In accordance with this aspect of the invention there are provided isolated nucleic acid molecules encoding greA, particularly *Streptococcus* greA, including mRNAs, cDNAs, genomic DNAs and, in further embodiments of this aspect of the invention include biologically, diagnostically, prophylactically, clinically or therapeutically useful variants, analogs or derivatives thereof, or fragments thereof, including fragments of the variants, analogs and derivatives, and compositions comprising same.

In accordance with another aspect of the present invention, there is provided the use of a polynucleotide of the invention for therapeutic or prophylactic purposes: in particular genetic immunization.

Among the particularly preferred embodiments of this aspect of the invention are naturally occurring allelic variants of greA and polypeptides encoded thereby.

In accordance with this aspect of the invention there are provided novel polypeptides of *Streptococcal* greA as well as biologically, diagnostically, prophylactically, clinically or therapeutically useful fragments, variants and derivatives thereof, variants and derivatives of the fragments, and analogs of the foregoing, and compositions comprising same.

Among the particularly preferred embodiments of this aspect of the invention are variants of greA polypeptide encoded by naturally occurring alleles of the greA gene.

In a preferred embodiment of this aspect of the invention there are provided methods for producing the aforementioned greA polypeptides.

In accordance with yet another aspect of the present invention, there are provided inhibitors to such polypeptides, useful as antibacterial agents, including, for example, antibodies.

In accordance with certain preferred embodiments of this aspect of the invention, there are provided products, compositions and methods, *inter alia*: assessing greA expression, to treat, for example, otitis media, conjunctivitis, pneumonia, bacteremia, meningitis, sinusitis, pleural empyema and endocarditis, and most particularly meningitis, such as for example infection of cerebrospinal fluid; assaying genetic variation; and administering a greA polypeptide or polynucleotide to an organism to raise an immunological response against a bacteria, especially a *Streptococcus*.

In accordance with certain preferred embodiments of this and other aspects of the invention there are provided polynucleotides that hybridize to greA polynucleotide sequences.

In certain additional preferred embodiments of this aspect of the invention there are provided antibodies against greA polypeptides.

In accordance with another aspect of the present invention, there are provided greA agonists which are also preferably bacteriostatic or bacteriocidal.

In accordance with yet another aspect of the present invention, there are provided greA antagonists which are also preferably bacteriostatic or bacteriocidal.

In a further aspect of the invention there are provided compositions comprising a greA polynucleotide or a greA polypeptide for administration to a cell or to a multicellular organism.

Various changes and modifications within the spirit and scope of the disclosed invention will become readily apparent to those skilled in the art from reading the following description and from reading the other parts of the present disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings depict certain embodiments of the invention. They are illustrative only and do not limit the invention otherwise disclosed herein.

Figure 1 shows a novel polynucleotide sequence of *Streptococcus pneumoniae* greA [SEQ ID NO:1]. The start codon (ATG) is shown in bold.

Figure 2 shows a novel amino acid sequence of *Streptococcus pneumoniae* greA [SEQ ID NO:2] deduced from the polynucleotide sequence of Figure 1.

GLOSSARY

The following illustrative explanations are provided to facilitate understanding of certain terms used frequently herein, particularly in the Examples. The explanations are provided as a convenience and are not limitative of the invention.

greA-BINDING MOLECULE, as used herein, refers to molecules or ions which bind or interact specifically with greA polypeptides or polynucleotides of the present invention, including, for example enzyme substrates, cell membrane components and classical receptors. Binding between polypeptides of the invention and such molecules, including binding or binding or interaction molecules may be exclusive to polypeptides of the invention, which is preferred, or it may be highly specific for polypeptides of the invention, which is also preferred, or it may be highly specific to a

group of proteins that includes polypeptides of the invention, which is preferred, or it may be specific to several groups of proteins at least one of which includes a polypeptide of the invention. Binding molecules also include antibodies and antibody-derived reagents that bind specifically to polypeptides of the invention.

GENETIC ELEMENT generally means a polynucleotide comprising a region that encodes a polypeptide or a polynucleotide region that regulates replication, transcription or translation or other processes important to expression of the polypeptide in a host cell, or a polynucleotide comprising both a region that encodes a polypeptide and a region operably linked thereto that regulates expression. Genetic elements may be comprised within a vector that replicates as an episomal element, that is, as a molecule physically independent of the host cell genome. They may be comprised within plasmids. Genetic elements also may be comprised within a host cell genome, not in their natural state but, rather, following manipulation such as isolation, cloning and introduction into a host cell in the form of purified DNA or in a vector, among others.

HOST CELL is a cell which has been transformed or transfected, or is capable of transformation or transfection by an exogenous polynucleotide sequence.

IDENTITY or SIMILARITY, as known in the art, are relationships between two or more polypeptide sequences or two or more polynucleotide sequences, as the case may be, as determined by comparing the sequences. In the art, identity also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. Both identity and similarity can be readily calculated (*Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Bio-computing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data*, Part I, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). While there exist a number of methods to measure identity and similarity between two polynucleotide or two polypeptide sequences, both terms are well known to skilled artisans (*Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., *SIAM J. Applied Math.*, 48: 1073 (1988). Methods commonly employed to determine identity or similarity between sequences include but are not limited to those disclosed in Carillo, H., and Lipman, D., *SIAM J. Applied Math.*, 48: 1073 (1988). Preferred methods to determine identity are designed to give the largest match between the sequences tested. Methods to determine identity and similarity are codified in computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCG program package (Devereux, J., et al., *Nucleic Acids Research* 12(1): 387 (1984)), BLASTP, BLASTN, and FASTA (Atschul, S.F. et al., *J. Molec. Biol.* 215: 403 (1990)).

ISOLATED means altered "by the hand of man" from its natural state; i.e., that, if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a naturally occurring polynucleotide or a polypeptide naturally present in a living organism in its natural state is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. As part of or following isolation, such polynucleotides can be joined to other polynucleotides, such as DNAs, for mutagenesis, to form fusion proteins, and for propagation or expression in a host, for instance. The isolated polynucleotides, alone or joined to other polynucleotides such as vectors, can be introduced into host cells, in culture or in whole organisms. Introduced into host cells in culture or in whole organisms, such DNAs still would be isolated, as the term is used herein, because they would not be in their naturally occurring form or environment. Similarly, the polynucleotides and polypeptides may occur in a composition, such as a media formulations, solutions for introduction of polynucleotides or polypeptides, for example, into cells, compositions or solutions for chemical or enzymatic reactions, for instance, which are not naturally occurring compositions, and, therein remain isolated polynucleotides or polypeptides within the meaning of that term as it is employed herein.

POLYNUCLEOTIDE(S) generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as used herein refers to, among others, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions or single-, double- and triple-stranded regions, single- and double-stranded RNA and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded, or triple-stranded, or a mixture of single- and double-stranded regions. In addition, polynucleotide as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide. As used herein, the term polynucleotide includes DNAs or RNAs as described above that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein.

it will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term polynucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells. *inter alia*, Polynucleotides embraces short polynucleotides often referred to as oligonucleotide(s).

POLYPEPTIDES, as used herein, includes all polypeptides as described below. The basic structure of polypeptides is well known and has been described in innumerable textbooks and other publications in the art. In this context, the term is used herein to refer to any peptide or protein comprising two or more amino acids joined to each other in a linear chain by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chain, which generally are referred to in the art as proteins, of which there are many types. It will be appreciated that polypeptides often contain amino acids other than the 20 amino acids commonly referred to as the 20 naturally occurring amino acids, and that many amino acids, including the terminal amino acids, may be modified in a given polypeptide, either by natural processes, such as processing and other post-translational modifications, but also by chemical modification techniques which are well known to the art. Even the common modifications that occur naturally in polypeptides are too numerous to list exhaustively here, but they are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature, and they are well known to those of skill in the art. Among the known modifications which may be present in polypeptides of the present are, to name an illustrative few, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, demethylation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination. Such modifications are well known to those of skill and have been described in great detail in the scientific literature. Several particularly common modifications, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, for instance, are described in most basic texts such as, for instance *PROTEINS-STRUCTURE AND MOLECULAR PROPERTIES*, 2nd Ed. T. E. Creighton, W. H. Freeman and Company, New York (1993). Many detailed reviews are available on this subject, such as, for example, those provided by Wold, F. Posttranslational Protein Modifications: Perspectives and Prospects, pgs. 1-12 in *POSTTRANSLATIONAL COVALENT MODIFICATION OF PROTEINS*, B. C. Johnson, Ed., Academic Press, New York (1983); Seifter et al., *Meth. Enzymol.* 182:626-646 (1990) and Rattan et al., *Protein Synthesis: Posttranslational Modifications and Aging*, Ann. N. Y. Acad. Sci. 663: 48-62(1992). It will be appreciated, as is well known and as noted above, that polypeptides are not always entirely linear. For instance, polypeptides may be generally as a result of posttranslational events, including natural processing event and events brought about by human manipulation which do not occur naturally. Circular, branched and branched circular polypeptides may be synthesized by non-translation natural process and by entirely synthetic methods, as well. Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. In fact, blockage of the amino or carboxyl group in a polypeptide, or both, by a covalent modification, is common in naturally occurring and synthetic polypeptides and such modifications may be present in polypeptides of the present invention, as well. For instance, the amino terminal residue of polypeptides made in *E. coli* or other cells, prior to proteolytic processing, almost invariably will be N-formylmethionine. During post-translational modification of the peptide, a methionine residue at the NH₂ terminus may be deleted. Accordingly, this invention contemplates the use of both the methionine-containing and the methionineless amino terminal variants of the protein of the invention. The modifications that occur in a polypeptide often will be a function of how it is made. For polypeptides made by expressing a cloned gene in a host, for instance, the nature and extent of the modifications in large part will be determined by the host cell posttranslational modification capacity and the modification signals present in the polypeptide amino acid sequence. For instance, as is well known, glycosylation often does not occur in bacterial hosts such as, for example, *E. coli*. Accordingly, when glycosylation is desired, a polypeptide should be expressed in a glycosylating host, generally a eukaryotic cell. Insect cell often carry out the same posttranslational glycosylations as mammalian cells and, for this reason, insect cell expression systems have been developed to express efficiently mammalian proteins having native patterns of glycosylation, *inter alia*. Similar considerations apply to other modifications. It will be appreciated that the same type of modification may be present in the same or varying degree at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. In general, as used herein, the term polypeptide encompasses all such modifications, particularly those that are present in polypeptides synthesized recombinantly by expressing a polynucleotide in a host cell.

VARIANT(S) of polynucleotides or polypeptides, as the term is used herein, are polynucleotides or polypeptides that differ from a reference polynucleotide or polypeptide, respectively. Variants in this sense are described below and elsewhere in the present disclosure in greater detail. (1) A polynucleotide that differs in nucleotide sequence from

another, reference polynucleotide. Generally, differences are limited so that the nucleotide sequences of the reference and the variant are closely similar overall and, in many regions, identical. As noted below, changes in the nucleotide sequence of the variant may be silent. That is, they may not alter the amino acids encoded by the polynucleotide. Where alterations are limited to silent changes of this type a variant will encode a polypeptide with the same amino acid sequence as the reference. Also as noted below, changes in the nucleotide sequence of the variant may alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Such nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below. (2) A polypeptide that differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference and the variant are closely similar overall and, in many regions, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions, fusions and truncations, which may be present in any combination.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to novel greA polypeptides and polynucleotides, among other things, as described in greater detail below. In particular, the invention relates to polypeptides and polynucleotides of a novel greA gene of *Streptococcus pneumoniae*, which is related by amino acid sequence homology to *E. coli* greA polypeptide. The invention relates especially to greA having the nucleotide and amino acid sequences set out in Figure 1 [SEQ ID NO:1] and Figure 2 [SEQ ID NO:2] respectively, and to the greA nucleotide and amino acid sequences of the DNA in NCIMB Deposit No. 40794, which is herein referred to as "the deposited bacterial strain" or as the "DNA of the deposited bacterial strain." It will be appreciated that the nucleotide and amino acid sequences set out in Figures 1 [SEQ ID NO:1] and 2 [SEQ ID NO:2] were obtained by sequencing the DNA of the deposited bacterial strain. Hence, the sequence of greA of the deposited bacterial strain is controlling as to any discrepancies between it (and the sequence it encodes) and the sequences of Figure 1 [SEQ ID NO:1] and Figure 2 [SEQ ID NO:2].

Techniques are available to evaluate temporal gene expression in bacteria, particularly as it applies to viability under laboratory and host infection conditions. A number of methods can be used to identify genes which are essential to survival *per se*, or essential to the establishment/maintenance of an infection. Identification of expression of a sequence by one of these methods yields additional information about its function and permits the selection of such sequence for further development as a screening target. Briefly these approaches include:

1) Signature Tagged Mutagenesis (STM)

This technique is described by Hensel *et al.*, *Science* 269: 400-403(1995), the contents of which is incorporated by reference for background purposes. Signature tagged mutagenesis identifies genes necessary for the establishment/maintenance of infection in a given infection model.

The basis of the technique is the random mutagenesis of target organism by various means (e.g., transposons) such that unique DNA sequence tags are inserted in close proximity to the site of mutation. The tags from a mixed population of bacterial mutants and bacteria recovered from an infected hosts are detected by amplification, radiolabeling and hybridization analysis. Mutants attenuated in virulence are revealed by absence of the tag from the pool of bacteria recovered from infected hosts.

In *Streptococcus pneumoniae*, because the transposon system is less well developed, a more efficient way of creating the tagged mutants is to use the insertion-duplication mutagenesis technique as described by Morrison *et al.*, *J. Bacteriol.* 159:870 (1984) the contents of which is incorporated by reference for background purposes.

2) In Vivo Expression Technology (IVET)

This technique is described by Camilli *et al.*, *Proc. Nat'l. Acad. Sci. USA.* 91:2634-2638 (1994), the contents of which is incorporated by reference for background purposes. IVET identifies genes up-regulated during infection when compared to laboratory cultivation, implying an important role in infection. Sequences identified by this technique are implied to have a significant role in infection establishment/maintenance.

In this technique random chromosomal fragments of target organism are cloned upstream of a promoter-less reporter gene in a plasmid vector. The pool is introduced into a host and at various times after infection bacteria may be recovered and assessed for the presence of reporter gene expression. The chromosomal fragment carried upstream of an expressed reporter gene should carry a promoter or portion of a gene normally upregulated during infection. Sequencing upstream of the reporter gene allows identification of the up regulated gene.

3) Differential display

This technique is described by Chuang *et al.*, *J. Bacteriol.* 175:2026-2036 (1993), the contents of which is incorporated by reference for background purposes. This method identifies those genes which are expressed in an organism by identifying mRNA present using randomly-primed RT-PCR. By comparing pre-infection and post infection profiles, genes up and down regulated during infection can be identified and the RT-PCR product sequenced and matched to library sequences.

4) Generation of conditional lethal mutants by transposon mutagenesis.

This technique, described by de Lorenzo, V. *et al.*, *Gene* 123:17-24(1993); Neuwald, A. F. *et al.*, *Gene* 125:69-73 (1993); and Takiff, H. E. *et al.*, *J. Bacteriol.* 174:1544-1553(1992), the contents of which is incorporated by reference for background purposes, identifies genes whose expression are essential for cell viability.

In this technique transposons carrying controllable promoters, which provide transcription outward from the transposon in one or both directions, are generated. Random insertion of these transposons into target organisms and subsequent isolation of insertion mutants in the presence of inducer of promoter activity ensures that insertions which separate promoter from coding region of a gene whose expression is essential for cell viability will be recovered. Subsequent replica plating in the absence of inducer identifies such insertions; since they fail to survive. Sequencing of the flanking regions of the transposon allows identification of site of insertion and identification of the gene disrupted. Close monitoring of the changes in cellular processes/morphology during growth in the absence of inducer yields information on likely function of the gene. Such monitoring could include flow cytometry (cell division, lysis, redox potential, DNA replication), incorporation of radiochemically labeled precursors into DNA, RNA, protein, lipid, peptidoglycan, monitoring reporter enzyme gene fusions which respond to known cellular stresses.

5) Generation of conditional lethal mutants by chemical mutagenesis.

This technique is described by Beckwith, J., *Methods in Enzymology* 204: 3-18(1991), the contents of which are incorporated herein by reference for background purposes. In this technique random chemical mutagenesis of target organism growth at temperature other than physiological temperature (permissive temperature) and subsequent replica plating and growth at different temperature (e.g. 42°C to identify ts, 25°C to identify cs) are used to identify those isolates which now fail to grow (conditional mutants). As above close monitoring of the changes upon growth at the non-permissive temperature yields information on the function of the mutated gene. Complementation of conditional lethal mutation by library from target organism and sequencing of complementing gene allows matching with library sequences.

Each of these techniques may have advantages or disadvantage depending on the particular application. The skilled artisan would choose the approach that is the most relevant with the particular end use in mind. For example some genes might be recognised as essential for infection but in reality are only necessary for the initiation of infection and so their products would represent relatively unattractive targets for antibacterials developed to cure established and chronic infections.

b) RT-PCR

Bacterial messenger RNA, preferably that of *Streptococcus pneumoniae*, is isolated from bacterial infected tissue e.g. 48 hour murine lung infections, and the amount of each mRNA species assessed by reverse transcription of the RNA sample primed with random hexanucleotides followed by PCR with gene specific primer pairs. The determination of the presence and amount of a particular mRNA species by quantification of the resultant PCR product provides information on the bacterial genes which are transcribed in the infected tissue. Analysis of gene transcription can be carried out at different times of infection to gain a detailed knowledge of gene regulation in bacterial pathogenesis allowing for a clearer understanding of which gene products represent targets for screens for novel antibacterials. Because of the gene specific nature of the PCR primers employed it should be understood that the bacterial mRNA preparation need not be free of mammalian RNA. This allows the investigator to carry out a simple and quick RNA preparation from infected tissue to obtain bacterial mRNA species which are very short lived in the bacterium (in the order of 2 minute halflives). Optimally the bacterial mRNA is prepared from infected murine lung tissue by mechanical disruption in the presence of TRIzol (GIBCO-BRL) for very short periods of time, subsequent processing according to the manufacturers of TRIzol reagent and DNAase treatment to remove contaminating DNA. Preferably the process is optimized by finding those conditions which give a maximum amount of bacterial 16S ribosomal RNA, preferably that of *Streptococcus pneumoniae*, as detected by probing Northernblots with a suitably labeled sequence specific oligonucleotide probe. Typically a 5' dye labelled primer is used in each PCR primer pair in a PCR reaction which is terminated

optimally between 8 and 25 cycles. The PCR products are separated on 6% polyacrylamide gels with detection and quantification using GeneScanner (manufactured by ABI).

Use of the of these technologies when applied to the sequences of the present invention enables identification of bacterial proteins expressed during infection, inhibitors of which would have utility in anti-bacterial therapy

Polynucleotides

In accordance with one aspect of the present invention, there are provided isolated polynucleotides which encode the greA polypeptide having the deduced amino acid sequence of Figure 2 [SEQ ID NO:2].

Using the information provided herein, such as the polynucleotide sequence set out in Figure 1 [SEQ ID NO:1], a polynucleotide of the present invention encoding greA polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning and sequencing chromosomal DNA fragments from *Streptococcus pneumoniae* 0100993 cells as starting material, followed by obtaining a full length clone. For example, to obtain a polynucleotide of the invention sequence, such as that sequence given in Figure 1 [SEQ ID NO:1] typically a library of clones of chromosomal DNA of *Streptococcus pneumoniae* 0100993 in *E. coli* or some other suitable host is probed with a radiolabeled oligonucleotide, preferably a 17-mer or longer, derived from a partial sequence. Clones carrying DNA identical to that of the probe can then be distinguished using high stringency washes. By sequencing the individual clones thus identified with sequencing primers designed from the original sequence it is then possible to extend the sequence in both directions to determine the full gene sequence. Conveniently such sequencing is performed using denatured double stranded DNA prepared from a plasmid clone. Suitable techniques are described by Maniatis, T., Fritsch, E.F. and Sambrook et al. *MOLECULAR CLONING, A LABORATORY MANUAL*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989). (see Screening By Hybridization 1.90 and Sequencing Denatured Double-Stranded DNA Templates 13.70). Illustrative of the invention, the polynucleotide set out in Figure 1 [SEQ ID NO:1] was discovered in a DNA library derived from *Streptococcus pneumoniae* 0100993.

greA of the invention is structurally related to other proteins of the greA/greB gene family, as shown by the results of sequencing the DNA encoding greA of the deposited bacterial strain. The DNA sequence thus obtained is set out in Figure 1 [SEQ ID NO:1]. It contains an open reading frame encoding a protein having about the number of amino acid residues set forth in Figure 2 [SEQ ID NO:2] with a deduced molecular weight that can be calculated using amino acid residue molecular weight values well known in the art. The protein exhibits greatest homology to *E. coli* greA protein among known proteins. greA of Figure 2 [SEQ ID NO:2] has about 42% identity over its entire length and about 63% similarity over its entire length with the amino acid sequence of *E. coli* greA.

Polynucleotides of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced by chemical synthetic techniques or by a combination thereof. The DNA may be doublestranded or single-stranded. Single-stranded DNA may be the coding strand also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

The coding sequence which encodes the polypeptide may be identical to the coding sequence of the polynucleotide shown in Figure 1 [SEQ ID NO:1]. It also may be a polynucleotide with a different sequence, which, as a result of the redundancy (degeneracy) of the genetic code, encodes the polypeptide of Figure 2 [SEQ ID NO:2].

Polynucleotides of the present invention which encode the polypeptide of Figure 2 [SEQ ID NO:2] may include, but are not limited to the coding sequence for the mature polypeptide, by itself; the coding sequence for the mature polypeptide and additional coding sequences, such as those encoding a leader or secretory sequence, such as a pre-, or pro- or prepro- protein sequence; the coding sequence of the mature polypeptide, with or without the aforementioned additional coding sequences, together with additional, non-coding sequences, including for example, but not limited to non-coding 5' and 3' sequences such as the transcribed, non-translated sequences that play a role in transcription (including termination signals, for example), ribosome binding, mRNA stability elements, and additional coding sequence which encode additional amino acids, such as those which provide additional functionalities. Thus for instance, the polypeptide may be fused to a marker sequence, such as a peptide, which facilitates purification of the fused polypeptide. In certain embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, such as the tag provided in the pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz et al., *Proc. Natl. Acad. Sci., USA* 86: 821-824 (1989) for instance, hexa-histidine provides for convenient purification of the fusion protein. The HA tag may also be used to create fusion proteins and corresponds to an epitope derived of influenza hemagglutinin protein, which has been described by Wilson et al., *Cell* 37: 767 (1984), for instance. Polynucleotides of the invention also include, but are not limited to, polynucleotides comprising a structural gene and its naturally associated genetic elements.

In accordance with the foregoing, the term "polynucleotide encoding a polypeptide" as used herein encompasses polynucleotides which include a sequence encoding a polypeptide of the present invention, particularly bacterial, and more particularly the *Streptococcus pneumoniae* greA having the amino acid sequence set out in Figure 2 [SEQ ID NO:2]. The term encompasses polynucleotides that include a single continuous region or discontinuous regions en-

coding the polypeptide (for example, interrupted by integrated phage or insertion sequence or editing) together with additional regions, that also may contain coding and/or non-coding sequences.

The present invention further relates to variants of the herein above described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figure 2 [SEQ ID NO: 2]. A variant of the polynucleotide may be a naturally occurring variant such as a naturally occurring allelic variant, or it may be a variant that is not known to occur naturally. Such non-naturally occurring variants of the polynucleotide may be made by mutagenesis techniques, including those applied to polynucleotides, cells or organisms.

Among variants in this regard are variants that differ from the aforementioned polynucleotides by nucleotide substitutions, deletions or additions. The substitutions, deletions or additions may involve one or more nucleotides. The variants may be altered in coding or noncoding regions or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions.

Among the particularly preferred embodiments of the invention in this regard are polynucleotides encoding polypeptides having the amino acid sequence of greA set out in Figure 2 [SEQ ID NO: 2]; variants, analogs, derivatives and fragments thereof, and fragments of the variants, analogs and derivatives.

Further particularly preferred in this regard are polynucleotides encoding greA variants, analogs, derivatives and fragments, and variants, analogs and derivatives of the fragments, which have the amino acid sequence of greA polypeptide of Figure 2 [SEQ ID NO: 2] in which several, a few, 5 to 10, 1 to 5, 1 to 3, 2, 1 or no amino acid residues are substituted, deleted or added, in any combination. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of greA. Also especially preferred in this regard are conservative substitutions. Most highly preferred are polynucleotides encoding polypeptides having the amino acid sequence of Figure 2 [SEQ ID NO: 2], without substitutions.

Further preferred embodiments of the invention are polynucleotides that are at least 70% identical over their entire length to a polynucleotide encoding greA polypeptide having the amino acid sequence set out in Figure 2 [SEQ ID NO: 2], and polynucleotides which are complementary to such polynucleotides. Alternatively most highly preferred are polynucleotides that comprise a region that is at least 80% identical over their entire length to a polynucleotide encoding greA polypeptide of the *Streptococcus pneumoniae* DNA of the deposited bacterial strain and polynucleotides complementary thereto. In this regard, polynucleotides at least 90% identical over their entire length to the same are particularly preferred, and among these particularly preferred polynucleotides, those with at least 95% are especially preferred. Furthermore those with at least 97% are highly preferred among those with at least 95%, and among these those with at least 98% and at least 99% are particularly highly preferred, with at least 99% being the more preferred.

Preferred embodiments in this respect, moreover, are polynucleotides which encode polypeptides which retain substantially the same biological function or activity as the mature polypeptide encoded by the DNA of Figure 1 [SEQ ID NO: 1].

The present invention further relates to polynucleotides that hybridize to the herein above-described sequences. In this regard, the present invention especially relates to polynucleotides which hybridize under stringent conditions to the herein above-described polynucleotides. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences.

As discussed additionally herein regarding polynucleotide assays of the invention, for instance, polynucleotides of the invention as discussed above, may be used as a hybridization probe for RNA, cDNA and genomic DNA to isolate full-length cDNAs and genomic clones encoding greA and to isolate cDNA and genomic clones of other genes that have a high sequence similarity to the greA gene. Such probes generally will comprise at least 15 bases. Preferably, such probes will have at least 30 bases and may have at least 50 bases. Particularly preferred probes will have at least 30 bases and will have 50 bases or less.

For example, the coding region of the greA gene may be isolated by screening using the known DNA sequence to synthesize an oligonucleotide probe. A labeled oligonucleotide having a sequence complementary to that of a gene of the present invention is then used to screen a library of cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

The polynucleotides and polypeptides of the present invention may be employed as research reagents and materials for discovery of treatments of and diagnostics for disease, particularly human disease, as further discussed herein relating to polynucleotide assays, *inter alia*.

The polynucleotides of the invention that are oligonucleotides, including SEQ ID NOS 3 and 4, derived from the sequences of SEQ ID NOS 1 and 2 may be used in the processes herein as described, but preferably for PCR, to determine whether or not the *Streptococcus pneumoniae* genes identified herein in whole or in part are transcribed in infected tissue. It is recognized that such sequences will also have utility in diagnosis of the stage of infection and type of infection the pathogen has attained.

The polynucleotides may encode a polypeptide which is the mature protein plus additional amino or carboxyl-terminal amino acids, or amino acids interior to the mature polypeptide (when the mature form has more than one polypeptide chain, for instance). Such sequences may play a role in processing of a protein from precursor to a mature

form, may allow protein transport, may lengthen or shorten protein half-life or may facilitate manipulation of a protein for assay or production, among other things. As generally is the case *in vivo*, the additional amino acids may be processed away from the mature protein by cellular enzymes.

A precursor protein, having the mature form of the polypeptide fused to one or more prosequences may be an inactive form of the polypeptide. When prosequences are removed such inactive precursors generally are activated. Some or all of the prosequences may be removed before activation. Generally, such precursors are called proproteins.

In sum, a polynucleotide of the present invention may encode a mature protein, a mature protein plus a leader sequence (which may be referred to as a preprotein), a precursor of a mature protein having one or more prosequences which are not the leader sequences of a preprotein, or a preproprotein, which is a precursor to a proprotein, having a leader sequence and one or more prosequences, which generally are removed during processing steps that produce active and mature forms of the polypeptide.

Deposited materials

The deposit has been made under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for Purposes of Patent Procedure. The strain will be irrevocably and without restriction or condition released to the public upon the issuance of a patent. The deposit is provided merely as convenience to those of skill in the art and is not an admission that a deposit is required for enablement, such as that required under 35 U.S.C. §112.

A deposit containing a *Streptococcus pneumoniae* bacterial strain has been deposited with the National Collections of Industrial and Marine Bacteria Ltd (NCIMB), 23 St. Machar Drive, Aberdeen AB2 1RY, Scotland on 11 April 1996 and assigned NCIMB Deposit No. 40794. The *Streptococcus pneumoniae* bacterial strain deposit is referred to herein as "the deposited bacterial strain" or as "the DNA of the deposited bacterial strain." The deposited material is a bacterial strain that comprises a full length *greA* gene.

The sequence of the polynucleotides contained in the deposited material, as well as the amino acid sequence of the polypeptide encoded thereby, are controlling in the event of any conflict with any description of sequences herein.

A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

Polypeptides

The present invention further relates to a *greA* polypeptide which has a deduced amino acid sequence of 160 amino acids in length, as set forth in Figure 2 [SEQ ID NO:2], and has a deduced molecular weight of 17 543 kilodaltons.

The invention also relates to fragments, analogs and derivatives of these polypeptides. The terms "fragment," "derivative" and "analog" when referring to the polypeptide of Figure 2 [SEQ ID NO:2], means a polypeptide which retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The fragment, derivative or analog of the polypeptide of Figure 2 [SEQ ID NO:2] may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol) or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

Among the particularly preferred embodiments of the invention in this regard are polypeptides having the amino acid sequence of *greA* set out in Figure 2 [SEQ ID NO:2] variants, analogs, derivatives and fragments thereof, and variants, analogs and derivatives of the fragments. Alternatively, particularly preferred embodiments of the invention in this regard are polypeptides having the amino acid sequence of the *greA*, variants, analogs, derivatives and fragments thereof, and variants, analogs and derivatives of the fragments.

Among preferred variants are those that vary from a reference by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics. Typically seen as conservative substitutions are the replacements one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg and replacements among the aromatic residues Phe, Tyr.

Further particularly preferred in this regard are variants, analogs, derivatives and fragments, and variants, analogs and derivatives of the fragments, having the amino acid sequence of the *greA* polypeptide of Figure 2 [SEQ ID NO:2], in which several, a few, 5 to 10, 1 to 5, 1 to 3, 2, 1 or no amino acid residues are substituted, deleted or added, in any

combination. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the greA. Also especially preferred in this regard are conservative substitutions. Most highly preferred are polypeptides having the amino acid sequence of Figure 2 [SEQ ID NO:2] without substitutions.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The polypeptides of the present invention include the polypeptide of Figure 2 [SEQ ID NO:2] (in particular the mature polypeptide) as well as polypeptides which have at least 70% identity to the polypeptide of Figure 2 [SEQ ID NO:2], preferably at least 80% identity to the polypeptide of Figure 2 [SEQ ID NO:2] and more preferably at least 90% similarity (more preferably at least 90% identity) to the polypeptide of Figure 2 [SEQ ID NO:2] and still more preferably at least 95% similarity (still more preferably at least 95% identity) to the polypeptide of Figure 2 [SEQ ID NO:2] and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length polypeptides. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

Fragments

Also among preferred embodiments of this aspect of the present invention are polypeptides comprising fragments of greA, most particularly fragments of greA having the amino acid set out in Figure 2 [SEQ ID NO:2], and fragments of variants and derivatives of the greA of Figure 2 [SEQ ID NO:2].

In this regard a fragment is a polypeptide having an amino acid sequence that entirely is the same as part but not all of the amino acid sequence of the aforementioned greA polypeptides and variants or derivatives thereof.

Such fragments may be "free-standing," *i.e.*, not part of or fused to other amino acids or polypeptides, or they may be comprised within a larger polypeptide of which they form a part or region. When comprised within a larger polypeptide, the presently discussed fragments most preferably form a single continuous region. However, several fragments may be comprised within a single larger polypeptide. For instance, certain preferred embodiments relate to a fragment of a greA polypeptide of the present comprised within a precursor polypeptide designed for expression in a host and having heterologous pre and pro-polypeptide regions fused to the amino terminus of the greA fragment and an additional region fused to the carboxyl terminus of the fragment. Therefore, fragments in one aspect of the meaning intended herein, refers to the portion or portions of a fusion polypeptide or fusion protein derived from greA.

Representative examples of polypeptide fragments of the invention, include, for example, fragments from amino acid number 1-20, 21-40, 41-60, 61-80, 81-100, and 101-120, 121-140, and 141-160, and any combination of these 20 amino acid fragments.

In this context "about" herein includes the particularly recited ranges larger or smaller by several, a few, 5, 4, 3, 2 or 1 amino acid at either extreme or at both extremes.

Preferred fragments of the invention include, for example, truncation polypeptides of greA. Truncation polypeptides include greA polypeptides having the amino acid sequence of Figure 2, or of variants or derivatives thereof except for deletion of a continuous series of residues (that is, a continuous region, part or portion) that includes the amino terminus, or a continuous series of residues that includes the carboxyl terminus or, as in double truncation mutants, deletion of two continuous series of residues, one including the amino terminus and one including the carboxyl terminus. Fragments having the size ranges set out about also are preferred embodiments of truncation fragments, which are especially preferred among fragments generally. Degradation forms of the polypeptides of the invention in a host cell, particularly a *Streptococcus*, are also preferred.

Also preferred in this aspect of the invention are fragments characterized by structural or functional attributes of greA. Preferred embodiments of the invention in this regard include fragments that comprise alpha-helix and alpha-helix forming regions, beta-sheet and beta-sheet-forming regions, turn and turn-forming regions, coil and coil-forming regions, hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions, substrate binding region, and high antigenic index regions of greA, and combinations of such fragments.

Preferred regions are those that mediate activities of greA. Most highly preferred in this regard are fragments that have a chemical, biological or other activity of greA, including those with a similar activity or an improved activity, or with a decreased undesirable activity. Further preferred polypeptide fragments are those that are antigenic or immunogenic in an animal, especially in a human.

It will be appreciated that the invention also relates to, among others, polynucleotides encoding the aforementioned fragments, polynucleotides that hybridize to polynucleotides encoding the fragments, particularly those that hybridize under stringent conditions, and polynucleotides, such as PCR primers, for amplifying polynucleotides that encode the

fragments. In these regards, preferred polynucleotides are those that correspond to the preferred fragments, as discussed above.

Vectors, host cells, expression

The present invention also relates to vectors which comprise a polynucleotide or polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells can be genetically engineered to incorporate polynucleotides and express polypeptides of the present invention. Introduction of a polynucleotide into the host cell can be affected by calcium phosphate transfection, DEAE-dextran mediated transfection, transfection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis et al., *BASIC METHODS IN MOLECULAR BIOLOGY*, (1986) and Sambrook et al., *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

Polynucleotide constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors or use with prokaryotic and eukaryotic hosts are described by Sambrook et al., *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

In accordance with this aspect of the invention the vector may be, for example, a plasmid vector, a single or double-stranded phage vector, a single or double-stranded RNA or DNA viral vector. Plasmids generally are designated herein by a lower case p preceded and/or followed by capital letters and/or numbers, in accordance with standard naming conventions that are familiar to those of skill in the art. Starting plasmids disclosed herein are either commercially available, publicly available, or can be constructed from available plasmids by routine application of well known, published procedures. Many plasmids and other cloning and expression vectors that can be used in accordance with the present invention are well known and readily available to those of skill in the art.

Preferred among vectors, in certain respects, are those for expression of polynucleotides and polypeptides of the present invention. Generally, such vectors comprise *cis*-acting control regions effective for expression in a host operatively linked to the polynucleotide to be expressed. Appropriate *trans*-acting factors either are supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

In certain preferred embodiments in this regard, the vectors provide for specific expression. Such specific expression may be inducible expression or expression only in certain types of cells or both inducible and cell-specific. Particularly preferred among inducible vectors are vectors that can be induced for expression by environmental factors that are easy to manipulate, such as temperature and nutrient additives. A variety of vectors suitable to this aspect of the invention, including constitutive and inducible expression vectors for use in prokaryotic and eukaryotic hosts, are well known and employed routinely by those of skill in the art.

A great variety of expression vectors can be used to express a polypeptide of the invention. Such vectors include, among others, chromosomal, episomal and virus-derived vectors, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids, all may be used for expression in accordance with this aspect of the present invention. Generally, any vector suitable to maintain, propagate or express polynucleotides to express a polypeptide in a host may be used for expression in this regard.

The appropriate DNA sequence may be inserted into the vector by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook et al., *MOLECULAR CLONING, A LABORATORY MANUAL*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

The DNA sequence in the expression vector is operatively linked to appropriate expression control sequence(s), including, for instance, a promoter to direct mRNA transcription. Representatives of such promoters include, but are not limited to, the phage lambda PL promoter, the *E. coli* lac, trp and tac promoters, the SV40 early and late promoters and promoters of retroviral LTRs.

In general, expression constructs will contain sites for transcription initiation and termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and a termination codon appropriately positioned at the end.

of the polypeptide to be translated

In addition, the constructs may contain control regions that regulate as well as engender expression. Generally, in accordance with many commonly practiced procedures, such regions will operate by controlling transcription, such as transcription factors, repressor binding sites and termination, among others.

Vectors for propagation and expression generally will include selectable markers and amplification regions, such as, for example, those set forth in Sambrook *et al.*, *MOLECULAR CLONING, A LABORATORY MANUAL*, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

Representative examples of appropriate hosts include bacterial cells, such as streptococci, staphylococci, *E. coli*, streptomycetes and *Bacillus subtilis* cells; fungal cells, such as yeast cells and *Aspergillus* cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS, HeLa, C 127, 3T3, BHK, 293 and Bowes melanoma cells; and plant cells.

The following vectors, which are commercially available, are provided by way of example. Among vectors preferred for use in bacteria are pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16A, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia, and pBR322 (ATCC 37017). Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVk3, pBPV, pMSG and pSVL available from Pharmacia. These vectors are listed solely by way of illustration of the many commercially available and well known vectors that are available to those of skill in the art for use in accordance with this aspect of the present invention. It will be appreciated that any other plasmid or vector suitable for, for example, introduction, maintenance, propagation or expression of a polynucleotide or polypeptide of the invention in a host may be used in this aspect of the invention.

Promoter regions can be selected from any desired gene using vectors that contain a reporter transcription unit lacking a promoter region, such as a chloramphenicol acetyl transferase ("CAT") transcription unit, downstream of restriction site or sites for introducing a candidate promoter fragment, i.e., a fragment that may contain a promoter. As is well known, introduction into the vector of a promoter-containing fragment at the restriction site upstream of the *cat* gene engenders production of CAT activity, which can be detected by standard CAT assays. Vectors suitable to this end are well known and readily available such as pKK232-8 and pCM7. Promoters for expression of polynucleotides of the present invention include not only well known and readily available promoters but also promoters that readily may be obtained by the foregoing technique using a reporter gene.

Among known prokaryotic promoters suitable for expression of polynucleotides and polypeptides in accordance with the present invention are the *E. coli* *lacI* and *lacZ* and promoters, the T3 and T7 promoters, the *gpt* promoter, the lambda PR, PL promoters and the *trp* promoter.

Among known eukaryotic promoters suitable in this regard are the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous sarcoma virus ("RSV"), and metallothionein promoters, such as the mouse metallothionein-I promoter.

Recombinant expression vectors will include, for example, origins of replication, a promoter preferably derived from a highly-expressed gene to direct transcription of a downstream structural sequence, and a selectable marker to permit isolation of vector containing cells after exposure to the vector.

Polynucleotides of the invention, encoding the heterologous structural sequence of a polypeptide of the invention generally will be inserted into the vector using standard techniques so that it is operably linked to the promoter for expression. The polynucleotide will be positioned so that the transcription start site is located appropriately 5' to a ribosome binding site. The ribosome binding site will be 5' to the AUG that initiates translation of the polypeptide to be expressed. Generally, there will be no other open reading frames that begin with an initiation codon, usually AUG, and lie between the ribosome binding site and the initiation codon. Also, generally there will be a translation stop codon at the end of the polypeptide and there will be a polyadenylation signal in constructs for use in eukaryotic hosts. Transcription termination signal appropriately disposed at the 3' end of the transcribed region may also be included in the polynucleotide construct.

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment appropriate secretion signals may be incorporated into the expressed polypeptide. These signals may be endogenous to the polypeptide or they may be heterologous signals.

The polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals but also additional heterologous functional regions. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N- or C-terminus of the polypeptide to improve stability and persistence in the host cell, during purification or during subsequent handling and storage. Also, region also may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability or to facilitate purification, among others, are familiar and routine techniques in the art. A preferred fusion protein comprises a heterologous region from immunoglobulin that is useful to solubilize or purify polypeptides. For example, EP-A-0 464 533 (Canadian counterpart 2045869) discloses fusion proteins comprising various portions of constant region of

immunoglobulin molecules together with another protein or part thereof. In drug discovery, for example, proteins have been fused with antibody Fc portions for the purpose of high-throughput screening assays to identify antagonists. See, D. Bennett *et al.*, *Journal of Molecular Recognition*, Vol. 8 52-58 (1995) and K. Johanson *et al.*, *The Journal of Biological Chemistry*, Vol. 270, No. 16 pp 9459-9471 (1995)

Cells typically then are harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification.

Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents. Such methods are well known to those skilled in the art.

Mammalian expression vectors may comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation regions, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking non-transcribed sequences that are necessary for expression.

greA polypeptide can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography is employed for purification. Well known techniques for refolding protein may be employed to regenerate active conformation when the polypeptide is denatured during isolation and/or purification.

Polynucleotide assays

This invention is also related to the use of the greA polynucleotides to detect complementary polynucleotides such as, for example, as a diagnostic reagent. Detection of greA in a eukaryote, particularly a mammal, and especially a human, will provide a diagnostic method for diagnosis of a disease. Eukaryotes (herein also "individual(s)"), particularly mammals, and especially humans, infected with an organism comprising the greA gene may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from an infected individual's cells and tissues, such as bone, blood, muscle, cartilage, and skin. Genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki *et al.*, *Nature*, 324: 163-166 (1986)) prior to analysis. RNA or cDNA may also be used in the same ways. As an example, PCR primers complementary to the nucleic acid encoding greA can be used to identify and analyze greA presence and/or expression. Using PCR, characterization of the strain of prokaryote present in a eukaryote, particularly a mammal, and especially a human, may be made by an analysis of the genotype of the prokaryote gene. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the genotype of a reference sequence. Point mutations can be identified by hybridizing amplified DNA to radiolabeled greA RNA or alternatively, radiolabeled greA antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Sequence differences between a reference gene and genes having mutations also may be revealed by direct DNA sequencing. In addition, cloned DNA segments may be employed as probes to detect specific DNA segments. The sensitivity of such methods can be greatly enhanced by appropriate use of PCR or another amplification method. For example, a sequencing primer is used with double-stranded PCR product or a single-stranded template molecule generated by a modified PCR. The sequence determination is performed by conventional procedures with radiolabeled nucleotide or by automatic sequencing procedures with fluorescent tags.

Genetic characterization based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels, with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, *e.g.*, Myers *et al.*, *Science*, 230: 1242 (1985)).

Sequence changes at specific locations also may be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (*e.g.*, Cotton *et al.*, *Proc. Natl. Acad. Sci., USA*, 85: 4397-4401 (1988)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, *e.g.*, restriction fragment length polymorphisms (RFLP) and Southern blotting of genomic DNA.

In addition to more conventional gel-electrophoresis and DNA sequencing, mutations also can be detected by *in situ* analysis.

Cells carrying mutations or polymorphisms in the gene of the present invention may also be detected at the DNA level by a variety of techniques, to allow for serotyping, for example. For example, RT-PCR can be used to detect mutations. It is particularly preferred to use RT-PCR in conjunction with automated detection systems, such as, for

example, GeneScan RNA or cDNA may also be used for the same purpose, PCR or RT-PCR. As an example, PCR primers complementary to the nucleic acid encoding greA can be selected and made using known methods and used to identify and analyze mutations. These primers may be used for amplifying greA DNA isolated from a sample derived from an individual. The invention also provides these primers with 1, 2, 3 or 4 nucleotides removed from the 5' and/or the 3' end. The primers may be used to amplify the gene isolated from the individual such that the gene may then be subject to various techniques for elucidation of the DNA sequence. In this way, mutations in the DNA sequence may be diagnosed.

The invention provides a process for diagnosing disease, preferably bacterial infections, more preferably *Streptococcus pneumoniae*, and most preferably otitis media, conjunctivitis, pneumonia, bacteremia, meningitis, sinusitis, pleural empyema and endocarditis and most particularly meningitis, such as for example infection of cerebrospinal fluid, comprising determining from a sample derived from an individual a increased level of expression of polynucleotide having the sequence of Figure 1 [SEQ ID NO: 1]. Increased expression of greA polynucleotide can be measured using any one of the methods well known in the art for the quantitation of polynucleotides, such as, for example, PCR, RT-PCR, RNase protection, Northern blotting and other hybridization methods.

Polypeptide assays

The present invention also relates to a diagnostic assays such as quantitative and diagnostic assays for detecting levels of greA protein in cells and tissues, including determination of normal and abnormal levels. Thus, for instance, a diagnostic assay in accordance with the invention for detecting over-expression of greA protein compared to normal control tissue samples may be used to detect the presence of an infection, for example. Assay techniques that can be used to determine levels of a greA protein, in a sample derived from a host are well-known to those of skill in the art. Such assay methods include radioimmunoassays, competitive-binding assays, Western Blot analysis and ELISA assays. Among these ELISAs frequently are preferred. An ELISA assay initially comprises preparing an antibody specific to greA, preferably a monoclonal antibody. In addition a reporter antibody generally is prepared which binds to the monoclonal antibody. The reporter antibody is attached a detectable reagent such as radioactive, fluorescent or enzymatic reagent, in this example horseradish peroxidase enzyme.

Antibodies

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. The present invention includes, for examples monoclonal and polyclonal antibodies, chimeric, single chain and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library.

Antibodies generated against the polypeptides corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptides itself. In this manner, even a sequence encoding only a fragment of the polypeptides can be used to generate antibodies binding the whole native polypeptides. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide.

For preparation of monoclonal antibodies, any technique known in the art which provides antibodies produced by continuous cell line cultures can be used. Examples include various techniques, such as those in Kohler, G. and Milstein, C., *Nature* 256: 495-497 (1975); Kozbor *et al.*, *Immunology Today* 4: 72 (1983); Cole *et al.*, pg. 77-96 in *MONOCLONAL ANTIBODIES AND CANCER THERAPY*, Alan R. Liss, Inc. (1985).

Techniques described for the production of single chain antibodies (U.S. Patent No. 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention. Also, transgenic mice, or other organisms such as other mammals, may be used to express humanized antibodies to immunogenic polypeptide products of this invention.

Alternatively phage display technology could be utilized to select antibody genes with binding activities towards the polypeptide either from repertoires of PCR amplified v-genes of lymphocytes from humans screened for possessing anti-Fbp or from naive libraries (McCafferty, J. *et al.*, (1990), *Nature* 348, 552-554; Marks, J. *et al.*, (1992) *Biotechnology* 10, 779-783). The affinity of these antibodies can also be improved by chain shuffling (Clackson, T. *et al.*, (1991) *Nature* 352, 624-628).

If two antigen binding domains are present each domain may be directed against a different epitope - termed 'bispecific' antibodies.

The above-described antibodies may be employed to isolate or to identify clones expressing the polypeptide or purify the polypeptide of the present invention by attachment of the antibody to a solid support for isolation and/or purification by affinity chromatography.

Thus, among others, antibodies against greA may be employed to inhibit and/or treat infections, particularly bac-

terial infections and especially otitis media, conjunctivitis, pneumonia, bacteremia, meningitis, sinusitis, pleural empyema and endocarditis, and most particularly meningitis, such as for example infection of cerebrospinal fluid.

Polypeptide derivatives include antigenically, epitopically or immunologically equivalent derivatives which from a particular aspect of this invention. The term "antigenically equivalent derivative" as used herein encompasses a polypeptide or its equivalent which will be specifically recognised by certain antibodies which, when raised to the protein or polypeptide according to the present invention, interfere with the immediate physical interaction between pathogen and mammalian host. The term "immunologically equivalent derivative" as used herein encompasses a peptide or its equivalent which when used in a suitable formulation to raise antibodies in a vertebrate, the antibodies act to interfere with the immediate physical interaction between pathogen and mammalian host.

The polypeptide, such as an antigenically or immunologically equivalent derivative or a fusion protein thereof is used as an antigen to immunize a mouse or other animal such as a rat or chicken. The fusion protein may provide stability to the polypeptide. The antigen may be associated, for example by conjugation, with an immunogenic carrier protein for example bovine serum albumin (BSA) or keyhole limpet haemocyanin (KLH). Alternatively a multiple antigenic peptide comprising multiple copies of the protein or polypeptide, or an antigenically or immunologically equivalent polypeptide thereof may be sufficiently antigenic to improve immunogenicity so as to obviate the use of a carrier.

Preferably the antibody or derivative thereof is modified to make it less immunogenic in the individual. For example, if the individual is human the antibody may most preferably be "humanized", where the complementarity determining region(s) of the hybridoma-derived antibody has been transplanted into a human monoclonal antibody, for example as described in Jones P. et al. (1986), *Nature* **321**, 522-525 or Tempest et al. (1991) *Biotechnology* **9**, 266-273.

The use of a polynucleotide of the invention in genetic immunization will preferably employ a suitable delivery method such as direct injection of plasmid DNA into muscles (Wolff et al., *Hum Mol Genet* 1992, **1**, 363, Manthorpe et al., *Hum. Gene Ther.* 1993, **4**, 419), delivery of DNA complexed with specific protein carriers (Wu et al., *J Biol Chem* 1989, **264**, 16985), coprecipitation of DNA with calcium phosphate (Benvenisty & Reshef, *PNAS*, 1986, **83**, 9551), encapsulation of DNA in various forms of liposomes (Kaneda et al., *Science* 1989, **243**, 375), particle bombardment (Tang et al., *Nature* 1992, **356**, 152, Eisenbraun et al. *DNA Cell Biol* 1993, **12**, 791) and *in vivo* infection using cloned retroviral vectors (Seeger et al., *PNAS* 1984, **81**, 5849).

greA-binding molecules and assays

This invention also provides a method for identification of molecules, such as binding molecules, that bind greA or the RNA sequence or RNA structure that greA binds. Genes encoding proteins that bind greA, can be identified by numerous methods known to those of skill in the art, for example, northwestern blotting and FACS sorting. Such methods are described in many laboratory manuals such as, for instance, Coligan et al., *Current Protocols in Immunology* **1**(2): Chapter 5 (1991). Also, a labeled ligand can be photoaffinity linked to a cell extract.

Polypeptides of the invention also can be used to assess greA binding capacity of greA-binding molecules, in cells or in cell-free preparations.

Polypeptides of the invention may also be used to assess the binding of small molecule substrates and ligands in, for example, cells, cell-free preparations, chemical libraries, and natural product mixtures. These substrates and ligands may be natural substrates and ligands or may be structural or functional mimetics.

Antagonists and agonists - assays and molecules

The invention also provides a method of screening compounds to identify those which enhance (agonist) or block (antagonist) the action of greA polypeptides or polynucleotides, such as its interaction with greA-binding molecules, including for example, interaction of greA with DNA or RNA sequences.

For example, to screen for agonists or antagonists, a synthetic reaction mix, a cellular compartment, such as a membrane, cell envelope or cell wall, or a preparation of any thereof may be prepared from a cell that expresses a molecule that binds greA. The preparation is incubated with labeled greA in the absence or the presence of a candidate molecule which may be a greA agonist or antagonist. The ability of the candidate molecule to bind the binding molecule is reflected in decreased binding of the labeled ligand. Molecules which bind gratuitously, *i.e.*, without inducing the effects of greA on binding the greA binding molecule, are most likely to be good antagonists. Molecules that bind well and elicit effects that are the same as or closely related to greA are agonists.

greA-like effects of potential agonists and antagonists may be measured, for instance, by determining activity of a reporter system following interaction of the candidate molecule with a cell or appropriate cell preparation, and comparing the effect with that of greA or molecules that elicit the same effects as greA. Reporter systems that may be useful in this regard include but are not limited to colorimetric labeled substrate converted into product, a reporter gene that is responsive to changes in greA activity, and binding assays known in the art.

Another example of an assay for greA antagonists is a competitive assay that combines greA and a potential

antagonist with membrane-bound greA-binding molecules, recombinant greA binding molecules, natural substrates or ligands, or substrate or ligand mimetics, under appropriate conditions for a competitive inhibition assay, greA can be labeled, such as by radioactivity or a colorimetric compound, such that the number of greA molecules bound to a binding molecule or converted to product can be determined accurately to assess the effectiveness of the potential antagonist.

Potential antagonists include small organic molecules, peptides, polypeptides, polynucleotides and antibodies that bind to a polypeptide of the invention and thereby inhibit or extinguish its activity. Potential antagonists also may be small organic molecules, a polynucleotide structurally analogous or homologous to the site of greA binding to polynucleotide, peptide, a polypeptide such as a closely related protein or antibody that binds the same sites on a binding molecule, such as a binding molecule, without inducing greA-induced activities, thereby preventing the action of greA by excluding greA from binding.

Potential antagonists include a small molecule which binds to and occupies the binding site of the polypeptide thereby preventing binding to cellular binding molecules, such that normal biological activity is prevented. Examples of small molecules include but are not limited to small organic molecules, nucleotides, nucleotide-like molecules, peptides or peptide-like molecules.

Other potential antagonists include antisense molecules (see Okano, *J. Neurochem.* 56: 560 (1991), *OLIGODEOXYNUCLEOTIDES AS ANTISENSE INHIBITORS OF GENE EXPRESSION*, CRC Press, Boca Raton, FL (1988), for a description of these molecules).

Preferred potential antagonists include compounds related to and derivatives of greA.

In a particular aspect the invention provides the use of the polypeptide, polynucleotide or inhibitor of the invention to interfere with the initial physical interaction between a pathogen and mammalian host responsible for sequelae of infection. In particular the molecules of the invention may be used: i) in the prevention of adhesion of bacteria, in particular gram positive bacteria, to mammalian extracellular matrix proteins on in-dwelling devices or to extracellular matrix proteins in wounds, ii) to block prokaryotic transcript cleavage factor protein mediated mammalian cell invasion by, for example, initiating phosphorylation of mammalian tyrosine kinases (Rosenshine *et al.*, *Infect. Immun.* 60:2211 (1992); iii) to block bacterial adhesion between mammalian extracellular matrix proteins and bacterial prokaryotic transcript cleavage factor proteins which mediate tissue damage; iv) to block the normal progression of pathogenesis in infections initiated other than by the implantation of in-dwelling devices or by other surgical techniques.

Each of the DNA sequences provided herein may be used in the discovery and development of antibacterial compounds. The encoded protein upon expression can be used as a target for the screening of antibacterial drugs. Additionally the DNA sequences encoding the amino terminal regions of the encoded protein or Shine-Delgado or other translation facilitating sequences of the respective mRNA can be used to construct antisense sequences to control the expression of the coding sequence of interest.

The antagonists and agonists may be employed for instance to inhibit otitis media, conjunctivitis, pneumonia, bacteremia, meningitis, sinusitis, pleural empyema and endocarditis, and most particularly meningitis, such as for example infection of cerebrospinal fluid.

Vaccines

Another aspect of the invention relates to a method for inducing an immunological response in an individual, particularly a mammal which comprises inoculating the individual with greA, or a fragment or variant thereof, adequate to produce antibody to protect said individual from infection, particularly bacterial infection and most particularly Streptococcus infections. Yet another aspect of the invention relates to a method of inducing immunological response in an individual which comprises, through gene therapy, delivering gene encoding greA, or a fragment or a variant thereof, for expressing greA, or a fragment or a variant thereof *in vivo* in order to induce an immunological response to produce antibody to protect said individual from disease.

A further aspect of the invention relates to an immunological composition which, when introduced into a host capable of having induced within it an immunological response, induces an immunological response in such host to a greA or protein coded therefrom, wherein the composition comprises a recombinant greA or protein coded therefrom comprising DNA which codes for and expresses an antigen of said greA or protein coded therefrom.

The greA or a fragment thereof may be fused with co-protein which may not by itself produce antibodies, but is capable of stabilizing the first protein and producing a fused protein which will have immunogenic and protective properties. Thus fused recombinant protein, preferably further comprises an antigenic co-protein, such as Glutathione-S-transferase (GST) or beta-galactosidase, relatively large co-proteins which solubilise the protein and facilitate production and purification thereof. Moreover, the co-protein may act as an adjuvant in the sense of providing a generalized stimulation of the immune system. The co-protein may be attached to either the amino or carboxy terminus of the first protein.

Provided by this invention are composition, particularly vaccine compositions, and methods comprising the

polypeptides or polynucleotides of the invention and immunostimulatory DNA sequences, such as those described in Sato, Y. *et al* Science 273: 352(1996)

Also, provided by this invention are methods using the described polynucleotide or particular fragments thereof which have been shown to encode non-variable regions of bacterial cell surface proteins in DNA construct used in such genetic immunization experiments in animal models of infection with *Streptococcus pneumoniae* will be particularly useful for identifying protein epitopes able to provoke a prophylactic or therapeutic immune response. It is believed that this approach will allow for the subsequent preparation of monoclonal antibodies of particular value from the requisite organ of the animal successfully resisting or clearing infection for the development of prophylactic agents or therapeutic treatments of *Streptococcus pneumoniae* infection in mammals, particularly humans.

The polypeptide may be used as an antigen for vaccination of a host to produce specific antibodies which protect against invasion of bacteria, for example by blocking adherence of bacteria to damaged tissue. Examples of tissue damage include wounds in skin or connective tissue caused e.g. by mechanical, chemical or thermal damage or by implantation of indwelling devices, or wounds in the mucous membranes, such as the mouth, mammary glands, urethra or vagina.

The present invention also includes a vaccine formulation which comprises the immunogenic recombinant protein together with a suitable carrier. Since the protein may be broken down in the stomach, it is preferably administered parenterally, including, for example, administration that is subcutaneous, intramuscular, intravenous, or intradermal. Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the bodily fluid, preferably the blood, of the individual; and aqueous and non-aqueous sterile suspensions which may include suspending agents or thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example, sealed ampoules and vials and may be stored in a freeze-dried condition requiring only the addition of the sterile liquid carrier immediately prior to use. The vaccine formulation may also include adjuvant systems for enhancing the immunogenicity of the formulation, such as oil-in water systems and other systems known in the art. The dosage will depend on the specific activity of the vaccine and can be readily determined by routine experimentation.

While the invention has been described with reference to certain *greA* compounds, it is to be understood that this covers fragments of the naturally occurring protein and similar proteins with additions, deletions or substitutions which do not substantially affect the immunogenic properties of the recombinant protein.

30 Compositions

The invention also relates to compositions comprising the polynucleotide or the polypeptides discussed above or the agonists or antagonists. Thus, the polypeptides of the present invention may be employed in combination with a non-sterile or sterile carrier or carriers for use with cells, tissues or organisms such as a pharmaceutical carrier suitable for administration to a subject. Such compositions comprise, for instance, a media additive or a therapeutically effective amount of a polypeptide of the invention and a pharmaceutically acceptable carrier or excipient. Such carriers may include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol and combinations thereof. The formulation should suit the mode of administration.

40 Kits

The invention further relates to diagnostic and pharmaceutical packs and kits comprising one or more containers filled with one or more of the ingredients of the aforementioned compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, reflecting approval by the agency of the manufacture, use or sale of the product for human administration.

Administration

Polypeptides and other compounds of the present invention may be employed alone or in conjunction with other compounds, such as therapeutic compounds.

The pharmaceutical compositions may be administered in any effective, convenient manner including, for instance, administration by topical, oral, anal, vaginal, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes among others.

The pharmaceutical compositions generally are administered in an amount effective for treatment or prophylaxis of a specific indication or indications. In general, the compositions are administered in an amount of at least about 10 µg/kg body weight. In most cases they will be administered in an amount not in excess of about 8 mg/kg body weight per day. Preferably, in most cases, dose is from about 10 µg/kg to about 1 mg/kg body weight, daily. It will be appreciated

that optimum dosage will be determined by standard methods for each treatment modality and indication, taking into account the indication, its severity, route of administration, complicating conditions and the like.

In therapy or as a prophylactic, the active agent may be administered to an individual as an injectable composition, for example as a sterile aqueous dispersion, preferably isotonic.

Alternatively the composition may be formulated for topical application for example in the form of ointments, creams, lotions, eye ointments, eye drops, ear drops, mouthwash, impregnated dressings and sutures and aerosols, and may contain appropriate conventional additives, including, for example, preservatives, solvents to assist drug penetration, and emollients in ointments and creams. Such topical formulations may also contain compatible conventional carriers, for example cream or ointment bases, and ethanol or oleyl alcohol for lotions. Such carriers may constitute from about 1% to about 98% by weight of the formulation: more usually they will constitute up to about 80% by weight of the formulation.

For administration to mammals, and particularly humans, it is expected that the daily dosage level of the active agent will be from 0.01 mg/kg to 10 mg/kg, typically around 1 mg/kg. The physician in any event will determine the actual dosage which will be most suitable for an individual and will vary with the age, weight and response of the particular individual. The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

In-dwelling devices include surgical implants, prosthetic devices and catheters, i.e., devices that are introduced to the body of an individual and remain in position for an extended time. Such devices include, for example, artificial joints, heart valves, pacemakers, vascular grafts, vascular catheters, cerebrospinal fluid shunts, urinary catheters, continuous ambulatory peritoneal dialysis (CAPD) catheters, etc.

The composition of the invention may be administered by injection to achieve a systemic effect against relevant bacteria shortly before insertion of an in-dwelling device. Treatment may be continued after surgery during the in-body time of the device. In addition, the composition could also be used to broaden perioperative cover for any surgical technique to prevent *Streptococcus* wound infections.

Many orthopaedic surgeons consider that humans with prosthetic joints should be considered for antibiotic prophylaxis before dental treatment that could produce a bacteremia. Late deep infection is a serious complication sometimes leading to loss of the prosthetic joint and is accompanied by significant morbidity and mortality. It may therefore be possible to extend the use of the active agent as a replacement for prophylactic antibiotics in this situation.

In addition to the therapy described above, the compositions of this invention may be used generally as a wound treatment agent to prevent adhesion of bacteria to matrix proteins exposed in wound tissue and for prophylactic use in dental treatment as an alternative to or in conjunction with, antibiotic prophylaxis.

Alternatively, the composition of the invention may be used to bathe an indwelling device immediately before insertion. The active agent will preferably be present at a concentration of 1 µg/ml to 10mg/ml for bathing of wounds or indwelling devices.

A vaccine composition is conveniently in injectable form. Conventional adjuvants may be employed to enhance the immune response.

A suitable unit dose for vaccination is 0.5-5µg/kg of antigen, and such dose is preferably administered 1-3 times and with an interval of 1-3 weeks.

With the indicated dose range, no adverse toxicological effects will be observed with the compounds of the invention which would preclude their administration to suitable individuals.

The antibodies described above may also be used as diagnostic reagents to detect the presence of bacteria containing the prokaryotic transcript cleavage factor protein.

In order to facilitate understanding of the following example certain frequently occurring methods and/or terms will be described.

EXAMPLES

The present invention is further described by the following examples. These exemplification's, while illustrating certain specific aspects of the invention, do not portray the limitations or circumscribe the scope of the disclosed invention.

All examples were carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. Routine molecular biology techniques of the following examples can be carried out as described in standard laboratory manuals, such as Sambrook et al., *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.(1989).

Example 1 Library Production

The polynucleotide having the DNA sequence given in SEQ ID NO:1 was obtained from a library of clones of

chromosomal DNA of *Streptococcus pneumoniae* in *E. coli*. In some cases the sequencing data from two or more clones containing overlapping *Streptococcus pneumoniae* DNAs was used to construct the contiguous DNA sequence in SEQ ID NO 1. Libraries may be prepared by routine methods, for example.

5 Methods 1 and 2 below.

Total cellular DNA is isolated from *Streptococcus pneumoniae* 0100993 according to standard procedures and size-fractionated by either of two methods.

10 Method 1

Total cellular DNA is mechanically sheared by passage through a needle in order to size-fractionate according to standard procedures. DNA fragments of up to 11 kbp in size are rendered blunt by treatment with exonuclease and DNA polymerase, and EcoRI linkers added. Fragments are ligated into the vector Lambda ZapII that has been cut with
15 EcoRI, the library packaged by standard procedures and *E. coli* infected with the packaged library. The library is amplified by standard procedures.

Method 2

20 Total cellular DNA is partially hydrolyzed with a one or a combination of restriction enzymes appropriate to generate a series of fragments for cloning into library vectors (e.g., RsaI, PstI, AluI, BshI235I), and such fragments are size-fractionated according to standard procedures. EcoRI linkers are ligated to the DNA and the fragments then ligated into the vector Lambda ZapII that have been cut with EcoRI, the library packaged by standard procedures, and *E. coli*
25 infected with the packaged library. The library is amplified by standard procedures.

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SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: Gentry, Daniel

(ii) TITLE OF THE INVENTION: Novel greA

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: SmithKline Beecham Corporation

(B) STREET: 709 Swedeland Road

(C) CITY: King of Prussia

(D) STATE: PA

(E) COUNTRY: USA

(F) ZIP: 19406-0939

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Diskette

(B) COMPUTER: IBM Compatible

(C) OPERATING SYSTEM: DOS

(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE: 23-JAN-1997

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 60/029633

(B) FILING DATE: 25-OCT-1996

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Gimmi, Edward R

(B) REGISTRATION NUMBER: 38,391

(C) REFERENCE/DOCKET NUMBER: GM50003

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 610-270-4478

(B) TELEFAX: 610-270-5090

(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 794 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGAGGATGT TGCGATTGAT ACCAACATTG ATTCACCTTA TAATGTTTAT
 AAAAATGTAG 60
 GTCTCATGCC TGGTCCAGTC GATAGTCCAA GTCTTGGATG CGGATTGAGT
 CAAGCATCAA 120
 TCAAACCTAAG AGCGATAACT TTTACTTTGT AGCAGATGTC ACAGAAGGCA
 AGGTTTACTA 180
 TGCTAACAAT CAAGAAGACC ACGACCGCAA TGTCGCTGAA CATGTCAACA
 GCAAATTAAA 240
 CTAAACAAAC TAAATTATG TGATACTTCA CATAATTTTC TTTAGAAAAT
 ATTATCAGAA 300
 GAAAGTTGAG AAAAATGGCA GAAAAAACAT ATCCTATGAC CCTTGAGGAA
 AAGGAAAAAC 360
 TTGAAAAAGA ATTAGAAGAA TTGAAATTGG TTCGTCGACC AGAAGTGGTA
 GAACGCATTA 420
 AGATTGCCCC TTCATACGGT GACCTTTTCA AAAACAGTGA GTACGAAGCA
 GCTAAGGATG 480
 AACAAAGCCTT TGTCGAAGGA CAAATCTCTA GCTTAGAAAC AAAAATCCGC
 TATGCTGAAA 540

TCGTCAATAG CGACGCAGTT GCCCAGGACG AAGTAGCGAT TGGTAAAACA
 GTCACCATCC 600
 5 AAGAAATTGG TGAGGACGAA GAAGAAGTTT ATATTATCGT AGGTTCAGCT
 GGTGCGGATG 660
 CCTTTGCAGG TAAGGTTTCA AATGAAAGCC CAATTGGGCA GGCCTTGATT
 10 GGCAAGAAAA 720
 CAGGTGATAC AGCAACCATT GAAACGCCTG TTGGTAGCTA TGATGTAAAA
 ATCTTGAAGG 780
 TTGAAAAAAC AGCC
 15 794

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 160 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Glu Lys Thr Tyr Pro Met Thr Leu Glu Glu Lys Glu Lys
 Leu
 1 5 10 15
 Glu Lys Glu Leu Glu Glu Leu Lys Leu Val Arg Arg Pro Glu Val
 Val
 20 25 30
 Glu Arg Ile Lys Ile Ala Arg Ser Tyr Gly Asp Leu Ser Glu Asn
 Ser
 35 40 45
 Glu Tyr Glu Ala Ala Lys Asp Glu Gln Ala Phe Val Glu Gly Gln
 Ile
 50 55 60
 Ser Ser Leu Glu Thr Lys Ile Arg Tyr Ala Glu Ile Val Asn Ser
 Asp

65 70 75
 80
 5 Ala Val Ala Gln Asp Glu Val Ala Ile Gly Lys Thr Val Thr Ile
 Gln
 85 90 95
 10 Glu Ile Gly Glu Asp Glu Glu Glu Val Tyr Ile Ile Val Gly Ser
 Ala
 100 105 110
 15 Gly Ala Asp Ala Phe Ala Gly Lys Val Ser Asn Glu Ser Pro Ile
 Gly
 115 120 125
 20 Gln Ala Leu Ile Gly Lys Lys Thr Gly Asp Thr Ala Thr Ile Glu
 Thr
 130 135 140
 25 Pro Val Gly Ser Tyr Asp Val Lys Ile Leu Lys Val Glu Lys Thr
 Ala
 145 150 155
 30 160

Claims

- 35 1. An isolated polynucleotide comprising a member selected from the group consisting of:
 (a) a polynucleotide having at least a 70% identity to a polynucleotide encoding a polypeptide comprising
 amino acids 1 to 160 of SEQ ID NO:2;
 (b) a polynucleotide which is complementary to the polynucleotide of (a); and
 40 (c) a polynucleotide comprising at least 15 sequential bases of the polynucleotide of (a) or (b).
 2. The polynucleotide of Claim 1 wherein the polynucleotide is DNA.
 3. The polynucleotide of Claim 1 wherein the polynucleotide is RNA.
 45 4. The polynucleotide of Claim 2 comprising nucleotide 1 to 794 set forth in SEQ ID NO:1.
 5. The polynucleotide of Claim 2 comprising nucleotide 315 to 794 set forth in SEQ ID NO:1.
 50 6. The polynucleotide of Claim 2 which encodes a polypeptide comprising amino acid 1 to 160 of SEQ ID NO:2.
 7. An isolated polynucleotide comprising a member selected from the group consisting of:
 (a) a polynucleotide having at least a 70% identity to a polynucleotide encoding the same mature polypeptide
 55 expressed by the greA gene contained in NCIMB Deposit No. 40794,
 (b) a polynucleotide complementary to the polynucleotide of (a); and
 (c) a polynucleotide comprising at least 15 bases of the polynucleotide of (a) or (b).

8. A vector comprising the DNA of Claim 2.
9. A host cell comprising the vector of Claim 8.
- 5 10. A process for producing a polypeptide comprising: expressing from the host cell of Claim 9 a polypeptide encoded by said DNA.
11. A process for producing a cell which expresses a polypeptide comprising transforming or transfecting the cell with the vector of Claim 8 such that the cell expresses the polypeptide encoded by the cDNA contained in the vector.
- 10 12. A polypeptide comprising an amino acid sequence which is at least 70% identical to amino acid 1 to 160 of SEQ ID NO:2.
13. A polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:2.
- 15 14. An antibody against the polypeptide of claim 12.
15. An antagonist which inhibits the activity of the polypeptide of claim 12.
- 20 16. A method for the treatment of an individual having need of greA comprising: administering to the individual a therapeutically effective amount of the polypeptide of claim 12.
17. The method of Claim 16 wherein said therapeutically effective amount of the polypeptide is administered by providing to the individual DNA encoding said polypeptide and expressing said polypeptide *in vivo*.
- 25 18. A method for the treatment of an individual having need to inhibit greA polypeptide comprising: administering to the individual a therapeutically effective amount of the antagonist of Claim 15.
19. A process for diagnosing a disease related to expression of the polypeptide of claim 12 comprising: determining a nucleic acid sequence encoding said polypeptide.
- 30 20. A diagnostic process comprising: analyzing for the presence of the polypeptide of claim 12 in a sample derived from a host.
- 35 21. A method for identifying compounds which bind to and inhibit an activity of the polypeptide of claim 12 comprising: contacting a cell expressing on the surface thereof a binding for the polypeptide, said binding being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said binding, with a compound to be screened under conditions to permit binding to the binding; and determining whether the compound binds to and activates or inhibits the binding by detecting the presence or absence of a signal generated from the interaction of the compound with the binding.
- 40 22. A method for inducing an immunological response in a mammal which comprises inoculating the mammal with greA, or a fragment or variant thereof, adequate to produce antibody to protect said animal from disease.
- 45 23. A method of inducing immunological response in a mammal which comprises, through gene therapy, delivering gene encoding greA fragment or a variant thereof, for expressing greA, or a fragment or a variant thereof *in vivo* in order to induce an immunological response to produce antibody to protect said animal from disease.
- 50 24. An immunological composition comprising a DNA which codes for and expresses a greA polynucleotide or protein coded therefrom which, when introduced into a mammal, induces an immunological response in the mammal to a given greA polynucleotide or protein coded therefrom.

FIGURE 1. *greA* cloned DNA sequence [SEQ ID NO:1]

```
1  CTGAGGATGT TCGGATTGAT ACCAACATTG ATTACACCTTA TAAATGTTTAT
51  AAAAAATGTAG GTCTCATGCC TGGTCCAGTC GATAGTCCAA GTCCTGGATG
101 CGGATTGAGT CAAGCATCAA TCAAACATAAG AGGATAAAT TTTACTTTGT
151 AGCAGATGTC ACAGAAGGCA AGGTTTACTA TGCTAACAAAT CAAGAAGACC
201 ACGACCCGCAA TGTCGCTGAA CATGTCAACA GCAAAATTAAA CTAAACAAAC
251 TAAAATTATG TGATACTTCA CATAATTTTC TTTAGAAAAT ATTATCAGAA
301 GAAAGTTGAG AAAAAATGGCA GAAAAAACAT ATCCTATGAC CCTTGAGGAA
351 AAGGAAAAAC TTGAAAAAGA ATTAGAAGAA TTGAAATTGG TTCGTGACC
401 AGAAGTGGTA GAACGCATTA AGATTGCCCG TTCATACGGT GACCTTTCAG
451 AAACACAGTGA GTACGAAGCA GCTAAGGATG AACAAAGCCTT TGTCGAAGGA
```

Figure 1A

501 CAAATCTCTA GCTTAGAAAC AAAAATCCGC TATGCTGAAA TCGTCAATAG
551 CGACGCAGTT GCCCAGGACG AAGTAGCGAT TGGTAAACA GTCACCATCC
601 AAGAAATTGG TGAGGACGAA GAAGAAGTTT ATATTATCGT AGGTTCAGCT
651 GGTGCGGATG CCTTTGCAGG TAAGGTTTCA AATGAAAAGCC CAATTGGGCA
701 GCCCTTCATT GGCAAGAAAA CAGGTGATAC AGCAACCATT GAAACGCCTG
751 TTGGTAGCTA TGATGTAAAA ATCTTGAAGG TTGAAAAAAC AGCC

FIGURE 2. greA deduced amino acid sequence [SEQ ID NO:2]

1 MAEKTYPMTL EEKEKELEKEL EELKLVRRPE VVERIKIARS YGDLSENSEY
51 EAAKDEQAFV EGQISSLETK IRYAEIVNSD AVAQDEVAIG KTVTIQEIGE
101 DEEEVYIIIVG SAGADAFAGK VSNEPIGQA LIGKKTGDTA TIETPVGSYD
151 VKILKVEKTA

(19)



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(11)

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(54) **Streptococcus pneumoniae polypeptide greA**

(57) greA polypeptides and DNA (RNA) encoding such greA from streptococcus pneumoniae and a procedure for producing such polypeptides by recombinant techniques is disclosed. Also disclosed are methods for utilizing such greA for the treatment of infection, particularly bacterial infections. Antagonists against such greA and their use as a therapeutic to treat infections,

particularly bacterial infections are also disclosed. Also disclosed are diagnostic assays for detecting diseases related to the presence of greA nucleic acid sequences and the polypeptides in a host. Also disclosed are diagnostic assays for detecting polynucleotides encoding greA/greB gene family and for detecting the polypeptide in a host.

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Application Number

which under Rule 45 of the European Patent Convention EP 97 30 8508
shall be considered, for the purposes of subsequent
proceedings, as the European search report

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
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Y	J. SPARKOWSKI ET AL.: "The nucleotide sequence of greA, a suppressor gene that restores growth of an Escherichia coli RNA polymerase mutant at high temperature." NUCLEIC ACIDS RESEARCH, vol. 18, no. 21, 1990, page 6443 XP002170729 * the whole document *	1-14, 16-24	
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)
			C07K C12Q C12N A61K G01N
INCOMPLETE SEARCH			
<p>The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.</p> <p>Claims searched completely:</p> <p>Claims searched incompletely:</p> <p>Claims not searched:</p> <p>Reason for the limitation of the search:</p> <p>see sheet C</p>			
Place of search		Date of completion of the search	Examiner
THE HAGUE		2 July 2001	Hix, R
CATEGORY OF CITED DOCUMENTS			
<p>X : particularly relevant if taken alone</p> <p>Y : particularly relevant if combined with another document of the same category</p> <p>A : technological background</p> <p>O : non-written disclosure</p> <p>P : intermediate document</p> <p>T : theory or principle underlying the invention</p> <p>E : earlier patent document, but published on, or after the filing date</p> <p>D : document cited in the application</p> <p>L : document cited for other reasons</p> <p>& : member of the same patent family, corresponding document</p>			

EP0 FORM 1501 (03/82) (P04/Co.1)



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INCOMPLETE SEARCH
SHEET C

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Although claims 16 to 18, 22 and 23 are directed to a method of treatment of the human/animal body (Article 52(4) EPC), the search has been carried out and based on the alleged effects of the compound/composition.

Claim(s) searched incompletely:
15

Reason for the limitation of the search:

Claim 15 refers to an antagonist of the polypeptide of claim 12 without giving a true technical characterization. Moreover, no such specific compounds are defined in the application. In consequence, the scope of said claim is ambiguous and vague and its subject-matter is not sufficiently disclosed and supported (Art. 83 and 84 EPC.)
No meaningful search can be carried out for such purely speculative claims whose wording is a mere recitation of the result to be achieved.



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**ANNEX TO THE EUROPEAN SEARCH REPORT
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The members are as contained in the European Patent Office EDP file on
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02-07-2001

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